Induction of Apoptosis in 9-Nitrocamptothecin-treated DU145 Human Prostate Carcinoma Cells Correlates with \textit{de Novo} Synthesis of CD95 and CD95 Ligand and Down-Regulation of c-FLIP\textsubscript{short} ¹

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ABSTRACT

Stimulation of CD95 leads to oligomerization of this receptor and the recruitment of the Fas-associated death domain (FADD) and procaspase-8 to form the death-inducing signaling complex (DISC). Subsequent proteolytic activation of caspase-8 at the DISC leads to the activation of downstream caspases and execution of apoptosis. The anticancer drug 9-nitrocamptothecin (9NC) inhibits the nuclear enzyme topoisomerase I (Top1), an event followed by apoptosis of cancer cells. We investigated whether other mechanisms downstream of the DNA-Top1–9NC complexing step regulate the apoptotic ability of 9NC in DU145 cells. We demonstrate that induction of apoptosis in DU145 cells, upon exposure to 9NC, is associated with \textit{de novo} expression of CD95 and CD95L, suggesting that 9NC-induced apoptosis is mediated by the CD95 system. In this line, we observed early activation of caspase-3, -7, and -8, but not -1, -9, and -10. Moreover, 9NC treatment resulted in the dramatic down-regulation of c-FLIP\textsubscript{short} expression, but not that of c-FLIP\textsubscript{long} or FADD. Furthermore, incubation of DU145 cells with a neutralizing antibody (NOK-1) to CD95 or transient transfection of a c-FLIP\textsubscript{short} expression vector into DU145 cells partially abrogated 9NC-triggered apoptosis. We propose that 9NC triggers apoptosis by driving DU145 cells from a nonapoptotic status (c-FLIP\textsubscript{short}, high, CD95\textsubscript{low}, CD95L\textsubscript{low}) toward a proapoptotic status (c-FLIP\textsubscript{short}, low, CD95\textsubscript{high}, CD95L\textsubscript{high}). These findings indicate that in addition to a Top1-mediated effect, 9NC can additionally activate a CD95/CD95L-dependent apoptotic pathway.

INTRODUCTION

Like the parental compound, CPT,³ the derivative anticancer drug 9NC interferes with the mechanism of action of the nuclear enzyme Top1 (1–4). In general, Top1 introduces transient single-strand breaks in chromatin DNA and relaxes it from supercoiling (5). The CPT analogues bind to and stabilize Top1-DNA complexes and allow the single-strand breaks to be converted to double-strand breaks, thus resulting in the degradation of chromatin DNA and death by apoptosis of the treated cells (1–4). 9NC induces apoptosis in cultured cells that are tumorigenic when xenografted in immunodeficient athymic mice and has demonstrated unprecedented curative effectiveness against all human tumors, including prostate carcinoma, grown as xenografts in immunodeficient mice (reviewed in Refs. 6–8).

The execution of apoptosis triggered in mammalian cells by many compounds, including Top1 and -2 inhibitors, is regulated by the activation of procaspases (9, 10), which are proenzymes of the aspartate-specific cysteine protease family and are activated in a hierarchical manner (reviewed in Refs. 11–13). Members of the caspase family have been categorized as either initiators (caspase -2, -8, -9, and -10) or effectors (caspase -3, -6, and -7 and others), depending on whether the caspase activity occurs at the signaling (i.e., early) or execution (i.e., late) stage, respectively, of the apoptotic process (reviewed in Refs. 14–16).

Caspases can be activated by certain members of the nerve growth factor/tumor necrosis factor receptor family, termed death receptors. The death receptor CD95 (APO-1/Fas) mediates apoptosis in a variety of cell types (17, 18). Engagement of CD95 by CD95 ligand (CD95L) or by agonistic antibodies results in the onset of apoptosis in many cells (19, 20). Many chemotherapeutic drugs, such as CPT, cisplatin, and methotrexate, can enhance the expression of CD95L and/or CD95, thereby sensitizing various cells, including DU145, to CD95 death receptor-mediated apoptosis (21, 22). Furthermore, stimulation of CD95 with an appropriate ligand leads to the formation of the DISC, which includes the adaptor protein FADD and procaspase-8 (FADD-like interleukin-1-β-converting enzyme/MACH/MCH5; Refs. 23–25). Recruitment of procaspase-8 to the DISC is required for activation of this protease (22–27) and is the apical step in the cascade of caspase activation resulting in apoptosis. However, recruitment of procaspase-8 to the DISC can be antagonized by the cellular protein c-FLIP which is structurally similar to caspase-8 but lacks catalytic activity (28–30). Two c-FLIP protein forms exist, c-FLIP\textsubscript{long} and c-FLIP\textsubscript{short}, with molecular weights of 55,000 and 26,000, respectively (30). The role of c-FLIP in the process of apoptosis induction is controversial because both pro- (31–33) and antiapoptosis (29, 34, 35) functions have been described for this protein. c-FLIP expression can regulate the sensitivity of CD95-triggered cell death upon T-cell receptor stimulation (36), modulate tumor necrosis factor-related apoptosis-related ligand-induced apoptosis in melanoma cells (37, 38) or sensitize cells to CD95-induced apoptosis after exposure to metabolic inhibitors (39). In contrast, over-expression of c-FLIP can promote cell survival by activating the nuclear factor-κB and extracellular signal-regulated kinase signaling pathways (40, 41).

A role for caspases in the induction of apoptosis in prostate cancer cells has been reported, and it appears that the requirement for caspase activation depends on the human prostate cancer cell line and/or the agent used to initiate apoptosis. Thus, activation or overexpression of caspase-7 and -3 has been correlated with induction of apoptosis in hormone-dependent LNCaP (42, 43) and hormone-independent DU145 cells (44, 45). Moreover, it has been reported that DU145 cells that express CD95 are resistant to CD95-induced cell death, but CPT treatment sensitizes the cells to CD95-mediated apoptosis via pro-caspase activation without altering the levels of CD95 or CD95L (46).

To date, no studies have been conducted on caspase activation in DU145 cells treated with 9NC.

In this communication, we demonstrate that 9NC-induced apoptosis of human prostate carcinoma DU145 cells also induces \textit{de novo} synthesis of CD95L and CD95, but incubation of the...
cells with an antibody to CD95L or transient transfection with a c-FLIPshort expression vector partially abrogates 9NC-triggered apoptosis. Our results suggest that induction of CD95L and CD95 and down-regulation of c-FLIPshort are coordinated and important events in the enhanced sensitization of DU145 cells to apoptosis following treatment with 9NC.

MATERIALS AND METHODS

Materials. Clinical grade 9NC was a generous gift from SuperGen, Inc. (Dublin, CA) and was suspended in polyethylene glycol-200 to form a stock of 9NC that was divided into aliquots and stored at −20°C until used. Fetal bovine serum, apotinin, antipain, leupeptin, pepstatin A, chymostatin, and phenylmethylsulfonyl fluoride were obtained from Sigma Chemical Co. (St. Louis, MO). Protein quantification reagents were obtained from Bio-Rad Laboratories, Inc. (Hercules, CA), and enhanced chemiluminescence reagents for Western-blot analysis were from Amersham Life Science, Inc. (Arlington Heights, IL). The antibody to caspase-3 was obtained from Transduction Laboratories (Lexington, KY), the antibody to CD95 was from Santa Cruz Biotechnology (Santa Cruz, CA), the antibody to poly(ADP-ribose) polymerase was from Biomol (Plymouth Meeting, PA), the antibodies to procaspase-1 and -2 were from Oncogene Science (Cambridge, MA), and the antibodies to caspases -7, -9, -10, CD95L (NOK-1), and FADD were from PharMingen (San Diego, CA). The secondary antimonous IgG1 and IgG2B antibodies were from Southern Biotechnology (Birmingham, AL), and the antibody to FLAG was from Sigma Chemical Co. The antibodies to procaspase-8 (C15) and c-FLIP (NF6) have been described (47, 48). The antibody to protein p28Bap31 was kindly provided by Dr. Gordon Shore, McGill University (Montreal, Canada).

Cells. The DU145 human prostate carcinoma cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 50 units/ml streptomycin. The cell cultures were maintained in a humidified incubator at 37°C under 5% CO2. DU145 cells are highly tumorigenic when xenografted in immunodeficient mice (49).

Flow Cytometry Analysis. Fractions (i.e., percentage) of apoptotic cells were determined by analysis of the relative DNA content in the cells by a dual laser FACSCalibur flow cytometer (Becton Dickinson) and Cell Cycle Analysis software from Modfit LT (Verity Software House, Inc., Topsham, MN).

Western Blot Analysis. Cell extracts were prepared in a cell lysis buffer containing 50 mM Tris (pH 7.4); 150 mM NaCl; 0.1% Triton X-100; 0.1% NP40; 4 mM EDTA; 50 mM NaF; 0.1 mM Na2V; 1 mM DTT; 10 mg/ml each of the protease inhibitors antipain, leupeptin, pepstatin A, and chymostatin; and 50 mg/ml phenylmethylsulfonyl fluoride (50). Protein concentrations of cell extracts were determined by a detergent-based assay (Bio-Rad). Proteins were separated by SDS-PAGE and then electrophoretically transferred from the gel to nitrocellulose membranes (VWR). Proteins recognized by the antibodies were detected with enhanced chemiluminescence Western-blot reagents.

RT-PCR Assay for CD95L. Total RNA was isolated from DU145 cells treated with 9NC for various intervals. Subsequently, 1 µg of RNA was used for first-strand synthesis, which then served as a template for PCR reactions using a 1× Strata CDNA Synthesis Kit purchased from Boehringer Mannheim (Indianapolis, IN). SensiQuantitative RT-PCR analysis was performed with one-fifth of the total CDNA synthesized. The efficiency of the reverse transcription and the amount of RNA used in the RT-PCR reaction were verified by the detection of human β-actin with the primers 5’-TCTGACACGACCTCTCAATGACCTGCG-3’ and 5’-CGTCATACCTCCTTGCTGTGATCCACATCCTG-3’. The primers used to detect CD95L were 5’-CTACTGAGTGACACAGTGGCC-3’ and 5’-GGGTCTTCCCATTCCAGAGGC-3’. The PCR products were analyzed on a 1% agarose gels containing ethidium bromide.

Cell Transfection. DU145 cells were transiently transfected with the control vector pC3NA3 (Invitrogen, Carlsbad, CA) or the expression vectors for c-FLIPshort (pEF rs FLAG-c-FLIP) or pcDNA3-FLAG-c-FLIPshort expression vector, using the Trans Fast transfection reagent (Promega, Madison, WI) according to the protocol recommended by the manufacturer.

RESULTS

Processing of caspase-3, -7 and -8 in 9NC-treated DU145 Cells. We have previously shown that treatment of DU145 cells with 40 nM 9NC for 120 h results in an apoptotic fraction of ~30% in the culture (49). This result prompted us to investigate which major caspases are present and/or activated in these cells during the drug treatment. We initially observed by flow cytometry an apoptotic fraction of ~35% in cultures of DU145 cells treated with 50 nM 9NC for 48 h. Western blot analysis of whole cell lysates revealed that the expression of procaspase-1, -9 and -10 remained unchanged in 9NC-treated DU145 cells (Fig. 1A). We also examined the status of procaspase-8. Two isoforms of procaspase-8 are predominantly expressed, procaspase-8/a and procaspase-8/b (47), and the active enzyme, caspase-8, is a heterotetramer composed of two p18 and p10 subunits (25, 47). As shown in Fig. 1A, untreated DU145 cells contain the 55/53-kDa procaspase-8/a/b isoforms, but in 9NC-treated DU145 cells, there is a partial conversion to the p43 and p41 kDa intermediate cleavage products. This result was consistent with the limited apoptotic fraction (35%) observed in 9NC-treated DU145 cells.

It has been reported that procaspase-3 and -7 are physiological targets of activated caspase-8 (52). In agreement with these reports, the 32-kDa procaspase-3 and the 35-kDa procaspase-7 were present at reduced levels in 9NC-treated DU145 cells relative to the untreated cells (Fig. 1A). The reduction is assumed to result from the processing...
RT-PCR as described in Materials and Methods. A, NOK-1 antibody abrogates 9NC-induced apoptosis. DU145 cells were untreated (CTR) or treated with 9NC for 48 h (9NC), and treated with NOK-1 antibody for 48 h (NOK-1), plated in the presence of NOK-1 antibody for 24 h and then treated with 9NC for 48 h (NOK-1+9NC), or treated with IgG1 at the time of plating for 48 h (IgG1). Cells were harvested, and the apoptotic fraction (percentage) was measured by flow-activated cell-sorting analysis. The results are the average ± SD (bars) of three independent experiments.

9NC TRIGGERS CD95-MEDIATED APOPTOSIS

9NC Treatment Induces Expression of CD95. DU145 cells have been shown to be resistant to triggering of CD95-mediated apoptosis (48), which could be explained by the absence of an essential pro-apoptotic molecule such as FADD or CD95 itself. To investigate this, we prepared lysates from untreated cells and cells treated with 9NC for various periods of time and examined them for quantitative changes in CD95. Our results demonstrated a de novo expression of CD95 in the 9NC-treated cells and indicated that the level of expression was dependent on the period of drug treatment (Fig. 3A). It was also apparent that induction of CD95 expression took place prior to 3 h of drug treatment. High levels of CD95 were present after 6 h of 9NC treatment, with essentially no further quantitative changes in CD95 expression up to 24 h of drug treatment, whereas a small increase was observed at 48 h of drug treatment (Fig. 3B). Finally, lysates from untreated cells and cells treated with the drug for 48 h were examined for quantitative alterations of FADD. No significant alterations were observed in FADD of the 9NC-treated cells (Fig. 3A). The same membrane was also examined for levels of the control protein, actin, which were similar between untreated and drug-treated cells. Therefore, the nearly parallel induction of CD95L and CD95 is most likely responsible for caspase-8 activation and apoptosis after 9NC treatment of DU145 cells.

9NC Treatment Down-Regulates Expression of c-FLIPshort. Procaspase-8 recruitment to the DISC and subsequent activation of this proenzyme can also be controlled by the levels of c-FLIP (28–30). Overexpression of c-FLIP can block procaspase-8 processing and apoptosis (31, 32). c-FLIPshort was present at similar levels in both untreated and 9NC-treated cells, whereas c-FLIPlong was readily observed in untreated cells but was absent in drug-treated cells (Fig. 4A). p28Bap31, another caspase-8-binding protein (57) that was used as a control, was present at similar levels in both untreated and 9NC-treated cells (Fig. 4A), confirming that the down-regulation of these procaspases. We examined the time course for the activation of procaspase-3, -7, and -8 after treatment with 9NC. Our results show that substantial processing of procaspase-8 did not occur until 20 h after treatment with 9NC (Fig. 1B). In contrast, procaspase-3 and -7 were not processed during this time course of 9NC treatment (Fig. 1B). These data suggest that 9NC-triggered apoptosis in DU145 cells occurs via the processing of procaspase-8, -3 and -7 and that caspase-8 appears to be the apical mediator of this process.

Induction of CD95L in 9NC-treated DU145 Cells. Induction of CD95L by various chemotherapeutic agents has been associated with induction of caspase-8-triggered apoptosis of various malignant cell lines (22, 53–55). Accordingly, we investigated the expression of CD95L mRNA via semiquantitative RT-PCR in DU145 cells prior to and after treatment with 9NC. Our results demonstrated the absence of CD95L mRNA in untreated DU145 cells and DU145 cells treated for 1 and 3 h with 9NC, but the induction of CD95L mRNA was readily observed in 9NC-treated cells between 6 and 12 h (Fig. 2A). Thereafter, no significant changes were observed in the amount of CD95L mRNA in cells treated up to 48 h with 9NC (Fig. 2A). The unaltered expression of β-actin in untreated and 9NC-treated cells indicates that the 9NC effect was specific for CD95L mRNA and not attributable to a global alteration of gene expression (Fig. 2A).

The induction of CD95L by 9NC suggested that apoptosis may be triggered via this protein after interaction with its receptor, CD95. To test this hypothesis, we determined the survival of DU145 cells by flow cytometry after 48 h of exposure to 9NC in the absence or presence of the NOK-1 antibody, which recognizes and neutralizes both membrane and soluble forms of CD95L, thereby preventing interaction with CD95 (56). Our results show that the average apoptotic fraction present in three independent experiments was 3% in untreated DU145 cells, 41% in 9NC-treated cells, 9% in NOK-1-treated (2 μg/ml) cells, 17% in DU145 cells plated with NOK-1 then exposed to 9NC for 48 h, and 34% in cells incubated with an IgG1 isotype-matched antibody and treated with 9NC for 48 h (Fig. 2B). These results demonstrate that CD95 plays a role in 9NC-triggered apoptosis and that NOK-1 is able to partially abrogate this effect.

Fig. 2. Induction of CD95L and abrogation of apoptosis by NOK-1 antibody in 9NC-treated DU145 cells. A, RT-PCR analysis of CD95L mRNA expression. Total RNA was isolated from DU145 cells, treated with 9NC for the indicated hours (h), and used for RT-PCR as described in “Materials and Methods.” B, NOK-1 antibody abrogates 9NC-induced apoptosis. DU145 cells were untreated (CTR) or treated with 9NC for 48 h (9NC), and treated with NOK-1 antibody for 48 h (NOK-1), plated in the presence of NOK-1 antibody for 24 h and then treated with 9NC for 48 h (NOK-1+9NC), or treated with IgG1 at the time of plating for 48 h (IgG1). Cells were harvested, and the apoptotic fraction (percentage) was measured by flow-activated cell-sorting analysis. The results are the average ± SD (bars) of three independent experiments.
c-FLIP<sub>short</sub> was selective. The time-dependent down-regulation of c-FLIP<sub>short</sub> triggered by 9NC indicated that the levels of c-FLIP<sub>short</sub> started to decrease after 10 h of exposure to 9NC and were significantly diminished after 20 h (Fig. 4B). From these results we conclude that 9NC treatment specifically down-regulates c-FLIP<sub>short</sub> expression, resulting in sensitization of CD95-mediated apoptosis.

**Transient Overexpression of c-FLIP<sub>short</sub> Blocks 9NC-triggered Procaspase-8 Processing and Apoptosis.** To elucidate the role of c-FLIP<sub>short</sub> in 9NC-treated caspase-8 activation and apoptosis, we transiently overexpressed c-FLIP<sub>short</sub> in DU145 cells. Cell lysates were prepared from untreated, 9NC-treated DU145, c-FLIP<sub>short</sub>-transfected (c-FLIP<sub>short</sub>/DU145) and c-FLIP<sub>short</sub>/DU145 cells treated with 9NC and analyzed for the status of procaspase-8, c-FLIP<sub>short</sub>, the FLAG epitope tag, and actin. Procaspase-8 was extensively processed in 9NC-treated DU145 cells, but not in c-FLIP<sub>short</sub>/DU145 cells treated with 9NC (Fig. 5A). To demonstrate that the status of c-FLIP<sub>short</sub> levels correlated with the reduction of procaspase-8 processing and apoptosis in 9NC-treated cells, we performed Western-blot analysis to determine the status of c-FLIP<sub>short</sub> in the same samples. Our results show a dramatic reduction in the 26-kDa c-FLIP<sub>short</sub> in 9NC-treated DU145 cells (Fig. 5B). Furthermore, treatment of c-FLIP<sub>short</sub>/DU145 cells with 9NC for 24 h did not significantly reduce the levels of ectopically expressed c-FLIP<sub>short</sub>, but did significantly reduce the level of endogenous c-FLIP<sub>short</sub> (Fig. 5B). We confirmed the presence of c-FLIP<sub>short</sub> protein in DU145 cells by probing a Western blot that was a duplicate of the blot shown in Fig. 5B with an antibody to the FLAG tag (Fig. 5C). The apoptotic fraction of each treatment condition was measured by flow-activated cell-sensing analysis. Our results indicate that an apoptotic fraction of ~30% was present in 9NC-treated DU145 cells, whereas the apoptotic fraction was <5% in untreated DU145 cells, 12% in c-FLIP<sub>short</sub>/DU145 cells, and 18% in c-FLIP<sub>short</sub>/DU145 cells treated with 9NC for 24 h. An apoptotic fraction of <5% was also measured in DU145 cells transfected with the empty pcDNA3 vector (data not shown). Collectively, these results lead us to conclude that transient expression of c-FLIP<sub>short</sub> in DU145 cells partially abrogates 9NC-triggered processing of procaspase-8 and subsequent apoptosis.

**DISCUSSION**

In this report we have presented experimental evidence to demonstrate a novel regulatory mechanism involved in the induction of apoptosis in DU145 human prostate carcinoma cells after exposure to the anticancer drug 9NC. Our studies revealed that this mechanism intimately involves the CD95 death receptor pathway and occurs via the induction of CD95L and CD95 and the down-regulation of c-FLIP<sub>short</sub>. Incubation of 9NC-treated DU145 cells with a neutralizing antibody to CD95L or the transient overexpression of c-FLIP<sub>short</sub> in the DU145 cells confers partial protection against 9NC-triggered procaspase-8 activation and subsequent apoptosis.

The CD95/CD95L system plays a critical role in transmitting apoptotic signals triggered by chemotherapeutic agents (22, 53–55). In some experimental systems, anticancer drugs such as doxorubicin, methotrexate, or bleomycin can induce the up-regulation of membrane CD95 and induction of CD95L expression and thus activate a receptor/ligand paracrine mechanism that results in CD95-dependent apoptosis (56). These observations do not indicate a universal mechanism, however, because the induction of CD95L by the chemotherapeutic agents topotecan, etoposide, or doxorubicin does not correlate with CD95-mediated acute apoptosis induction in thymineless colon carcinoma cells (58). Furthermore, other studies have demonstrated that various agents induce procaspase-8 and -3 activation and apoptosis without altering the CD95 and CD95L levels in the drug-treated cells (56, 59).

The precise role of the regulation of the CD95/CD95L system is unclear in apoptosis induction in prostate cells. In this regard, it has been reported that the drug CPT sensitizes androgen-independent prostate cancer cells to anti-CD95-induced apoptosis, a result that could be blocked by the presence of the caspase inhibitor zVAD-fmk with no involvement of regulation of CD95 or CD95L (48). In other studies, the overexpression of mitogen-activated protein kinase phosphatase-1 rendered DU145 cells resistant to CD95L-mediated apoptosis (60), vector-expressed CD95L induced apoptosis in the androgen-independent PC3 prostate human cancer cell line (61), and induction of soluble CD95L accounted for the cytotoxicity of mitoxantrone in the androgen-dependent human prostate cancer cell line LNCaP (62). We observed significant induction of both CD95L and
CD95 in 9NC-treated DU145 cells (Figs. 2 and 3), indicating that in our cell system, 9NC-induced apoptosis does involve the regulation of CD95/CD95L. It is apparent from our results and reports from others (48, 53–56, 59, 61, 62) that the apoptotic response triggered by CD95 is dependent not only on the chemotherapeutic agent used but also on the in vitro experimental system. In this regard, it has been established that the effect of all CPT congeners on the primary target, the Top1/DNA complex, is very rapid and required for the appearance of subsequent events, such as the de novo synthesis of CD95 and CD95L, that lead to apoptosis. The mechanism(s) that may explain how inhibition of Top1 activates death systems such as CD95 is unknown at present.

It has also been reported that DU145 cells express CD95 and that CPT treatment sensitizes the cells to CD95-mediated apoptosis without altering the levels of CD95 or CD95L (48). Our results in the present study indicated a lack of CD95L or CD95 expression in untreated DU145 cells, but both of these molecules were detectable in 9NC-treated cells. It should be mentioned that the findings presented here were derived from cells originally obtained from American Type Culture Collection and used between approximately passages 60 and 70. However, these findings and the differential regulation of c-FLIP were barely detectable or undetectable in other “DU145 cell lines” obtained from other laboratories where they had been propagated for undetermined passages. We therefore would like to caution that the propagation passage, i.e., age of cultured cells, may be an important factor in the discrepancy of reported findings.

The ability of CPT (48) and 9NC (this study) to stimulate caspase-dependent apoptosis in DU145 cells corroborates the suggestion that activation of procaspase-8 is critical in the regulation of apoptosis signaling via CD95 (24, 25). This is a novel mechanism that may regulate the anticancer activity of 9NC, which has been associated with Top1 targeting (1, 2). In this regard, the Top1 inhibitor topotecan does not trigger apoptosis via CD95/CD95L in thymineless human colon carcinoma cells (58). In DU145 cells, however, NOK-1 partially abrogated 9NC-triggered apoptosis (Fig. 2), indicating that the 9NC-induced apoptosis signaling involves the CD95 pathway. The lack of complete inhibition of 9NC-triggered apoptosis in DU145 cells by NOK-1 suggests that 9NC may activate cell death by non-CD95 signaling pathways.

Overexpression of c-FLIP can block caspase-8 activation and CD95-mediated apoptosis in type I and II cells (30, 63). Cellular FLIPs resemble caspase-8 but lack proteolytic activity, are highly expressed in tumor cells and T lymphocytes, and have an integral role as endogenous modulators of apoptosis (64). The enhanced expression of c-FLIP in stably transfected Jurkat cells, however, did not prevent apoptosis induced by granzyme B in combination with adenovirus or sublytic concentrations of perforin (65). Furthermore, overexpression of c-FLIP did not prevent apoptosis in Jurkat cells after exposure to the DNA-damaging agents doxorubicin, etoposide, vincristine, and irradiation (66), indicating that apoptosis in Jurkat cells can proceed in a CD95-independent manner. Although both c-FLIP forms originate from the same gene by differential splicing, the precise relationship between c-FLIPshort and c-FLIPlong remains unclear. Metabolic inhibitors such as cycloheximide or actinomycin D have been shown to down-regulate c-FLIPshort, resulting in impaired retention of c-FLIP to the CD95 DISC (39), indicating that intracellular levels of one or both forms of c-FLIP determine the susceptibility of a cell to CD95-triggered apoptosis. In our study we observed the down-regulation of c-FLIPshort, but not c-FLIPlong in DU145 cells exposed to 9NC (Fig. 3), which demonstrates that these proteins are independently regulated during 9NC-induced apoptosis. Analysis of the kinetics of 9NC-triggered apoptosis implies that the down-regulation of c-FLIPshort is the major determinant of the events induced by 9NC.

Hence, CD95 and CD95L were fully up-regulated at a time point at which no apoptosis could be detected in DU145 cells. Only after the down-regulation of c-FLIPshort was apoptosis observed, which implies that c-FLIPshort expression is able to prevent apoptosis in 9NC-treated DU145 cells. It will be of interest to measure the kinetics of DISC formation upon direct CD95 triggering in untreated and 9NC-treated DU145 cells, i.e., whether 9NC-treated DU145 cells form DISC in the absence of CD95 triggering, which would indicate that endogenous CD95L is able to cross-link the receptor. The ability of transiently expressed c-FLIPshort to extensively inhibit 9NC-triggered caspase-8 activation (Fig. 5) implies an important regulatory role for c-FLIPshort during 9NC-triggered apoptosis in DU145 cells. This hypothesis is supported by the observations that c-FLIPshort diminishes prior to the activation of caspase-8 (Figs. 1 and 3) and that this event appears to be a prerequisite for activation of this caspase. These data imply that c-FLIPshort assumes an antiapoptotic function in DU145 cells and that abrogation of this function via 9NC treatment allows activation of procaspase-8.

To avoid inappropriate cell death and disease, the biochemical signals regulated by the death receptor signal need to be tightly regulated (67). c-FLIP is a protein that has the potential to control CD95-mediated apoptosis induction. Two reports strengthen this hypothesis and give new importance to the role of c-FLIP in the immune escape of tumors (68, 69). In murine tumors, the enhanced expression of c-FLIP results in escape from T-cell immunity, and this has been correlated with selection of tumor cells that overexpress c-FLIP (68). The Kaposi’s sarcoma-associated herpes virus protein-FLIP can act as a tumor progression factor by promoting tumor establishment and growth (69). When injected into immunocompetent recipient mouse strains, murine B-lymphoma cells transfected with Kaposi’s sarcoma-associated herpes virus protein-FLIP develop into tumors of high survival and growth (67, 69). Together these studies reveal the importance of CD95-mediated tumor cell death in vivo. In addition, FLIP expression is a mechanism that tumor cells can use to escape from T-cell immunity in vivo (70). Given these results, inhibition of c-FLIP expression may be a strategy to increase the efficacy of current chemotherapies (39, 67). The ability of 9NC to suppress the expression of c-FLIPshort in DU145 human prostate cancer cells clearly supports such a hypothesis. It will be of great interest to determine the status of c-FLIPshort and c-FLIPlong in human prostate cancer cells that are resistant to 9NC and to further elucidate the importance of this protein in CPT-based therapies.

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